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APPLIED RESEARCH CONCERNING ARTIFICIAL PHOTOSYNTHESIS

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(Prepared under Contract No. AF 33(616)-7255
by Jean A. Gross, Milton J. Becker, and Alan M. Shefner
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FOREWORD

This program was conducted under the sponsorship of the Life Support Systems Laboratory, 6570th Aerospace Medical Research Laboratories, Aerospace Medical Division, Wright-Patterson Air Force Base, Ohio. The work was carried out by Armour Research Foundation of Illinois Institute of Technology, Technology Center, Chicago 16, Illinois, under Contract No. AF 33(616)-7255, Project 6373, "Equipment for Life Support in Aerospace," and Task 637301, "Applied Ecology." The program was designated ARF Project C 174. This report covers the duration of the project, from May 1, 1960, to September 30, 1961.

The authors are J. A. Gross, Research Biochemist, Milton J. Becker, Associate Biochemist, and A. M. Shefner, Supervisor, Life Sciences Research. Dr. Gross was the project leader. Dr. Richard Ehrlich, Assistant Director of Life Sciences Research, provided invaluable administrative supervision and guidance.

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The original data was reported in ARF Reports 1 through 16.

Armour Research Foundation has designated their final report as ARF 3174-17.

ABSTRACT

The structure, biochemistry, and photosynthetic function of the spinach chloroplast is being investigated to gain sufficient knowledge to permit a similar active system to be synthesized by man.

Chloroplasts were isolated and fragmented. Relatively narrow-range size groupings of the fragments were obtained by differential centrifugation. These fractions were assayed physiologically and chemically. Maximal functional activity, in terms of electrons transferred or oxygen evolved under illumination, was found in particles sedimenting between 20 x 10^3 g and 80 x 10^3 g. Various anion concentrations were tested. An attempt to obtain photoactivity from a dried chloroplast layer -- a first approximation of an artificial system -- was only partially successful.

The studies indicate that activity in the fractions varies, probably reflecting altered structural or chemical composition. Differences in protein nitrogen concentration and chlorophyll:protein ratios are evidence of chemical changes. Results are discussed in relation to proposed models for the fine structure of the chloroplast and the basic photosynthetic unit.

PUBLICATION REVIEW

Wayne H. McCandless

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I. INTRODUCTION

The advent of the space age has stimulated investigations of various life-supporting systems which are mandatory for manned space flights. Space-weight relationships in manned spacecraft become especially significant with anticipated longer missions and larger crews. Presently employed chemical gas-exchange systems for carbon dioxide and oxygen turnover introduce serious problems of weight and space consumption. These problems might be overcome by the use of algal systems. However, the use of photosynthetic algae in a closed ecosystem is fraught with complications. The system is generally efficient only in relatively dilute solution. Bacterial contamination might disrupt the balance of or even destroy the system completely. Undesirable genetic changes, autointoxication, and engineering problems such as lighting, frothing, and positive thigmotaxis of the algae present difficulties for the practical application of a cyclic ecosystem. [6]

An artificial photosynthetic unit would not be subject to such problems. This is not to say that it would be inherently faultless, but rather that the obstacles are different. Such a synthetic system, if developed, could conceivably perform any or all of the activities of the photosynthetic system.

The ultimate aim of this program is to provide sufficient basic knowledge about the photosynthetic system to permit design and construction of an artificial reactor unit capable of simulating the process of photosynthesis, especially the gas-exchange cycle. Toward this end the chemistry, structure, and function of the plant photosynthetic organelle, the chloroplast, and its component parts are being studied.

There is a large volume of information in the literature concerning the biochemistry of photosynthesis and the structure of chloroplasts. Increasing numbers of studies in the biophysics and the energetics of the photoreaction are appearing. However, a basic structural-functional unit of photosynthesis has not been clearly defined. An optimal functional unit may require the combined or cooperative organization of a number of smaller units. The size of the optimal unit might also depend upon the specific part of the photosynthetic cycle being studied. Thus the optimal unit for photophosphorylation could be different from that for the Hill reaction. The Hill reaction is defined as the light-induced transfer of electrons or evolution of oxygen in the presence of an added hydrogen acceptor. Only by knowing the chemical composition and the structural organization of the functional units can a synthetic system be constructed.

Kupke and French[26] point out that attempts to prepare artificial photosynthetic systems have a relatively long history, dating from the investigations of Eisler and Portheim in 1922.[16] The most recent works of note are those of Rodrigo,[33] Sapozhnikov and Maslova,[35] and Vishniac.[42] All of these workers have recombined a protein with an organic solvent extract of chlorophyll and subsequently obtained a Hill reaction. This approach has had limited success but shows some promise. The experimental design in all cases has been an empirical one. Certainly the chance for successfully building an artificial photosynthetic structure should be greater if the nature of the building blocks and the blueprint for organizing them are first understood.

The approach used in this program, based to some extent upon the partial success of a similar attack upon mitochondria, is to isolate chloroplasts and fragment them. Successively smaller physical units are selected by differential centrifugation and then assayed for photoactivity. This study has led to the functional definition of particle size groups according to maximal and minimal activity. These groups are being studied for structural and chemical differences which might be correlated with activity. The composition and the molecular organization of the particles will also be investigated. Eventually, the accumulated factual information about photosynthesis can be applied to the problem of building a functional man-made facsimile of a gas-exchange system.

II. PREPARATION OF MATERIALS

The leaves of common spinach, Spinacia oleracea, served as the source of chloroplasts. Spinach was purchased as needed. The choice of spinach as the chloroplast source was based chiefly on three reasons: (1) many photosynthetic studies reported in the literature have employed this material, (2) it is readily available, and (3) chloroplasts can be isolated from spinach with relative ease.

Before a preparative procedure for obtaining the desired experimental samples was selected, a number of variables which could affect the final results were explored. These variables were conditions and duration of leaf storage, methods of cell rupture, solution composition, osmotic pressure, and conditions of chloroplast isolation and storage. The pertinent information which bears directly upon the reproducibility of results is included in Section IVA-1.

The procedure, which was developed, is schematized in Fig. 1 and is summarized below. Leaves were cut from the main portion of the spinach plant and washed for several hours in cold running tap water in a darkened room. The leaves were lightly blotted and stored in plastic bags in a refrigerator. Generally the leaves were used the following day, but in some cases as many as five or six days elapsed. In some cases, no significant difference in photoactivity was detected when leaves were stored up to 2 weeks.

All subsequent operations were performed under dim green light in an ice bath or in a semidarkened cold room at 4 °C. Spinach leaves were deribbed and blotted on paper toweling. Batches weighing 75 to 80 gms were minced, two to four batches being used per experiment. The minced leaf tissue was homogenized for two 15-sec intervals at top speed in a Waring blender in a volume of sucrose buffer equivalent to twice the tissue weight. The sucrose buffer contained 0.4 M sucrose, 0.05 M phosphate, and 0.01 M sodium chloride, pH 6.8. The homogenate was filtered through eight layers of cheesecloth and centrifuged for 5 min at 200 x g to remove cellular debris.

Chloroplasts were spun from the supernatant at 600 x g for 12 min in a refrigerated centrifuge. The sedimented chloroplasts were resuspended in the sucrose buffer and again centrifuged in order to wash out the majority of remaining smaller cytoplasmic particles. Leech and Ellis[27] used transaminase activity, which they associate with mitochondria, as a measure of the purity of a chloroplast preparation. They showed that one or two washings of a crude chloroplast suspension resulted in a low transaminase activity, indicating that most of the mitochondria which had been coprecipitated were removed.

We used a once-washed, intact chloroplast suspension (WCI) as the starting material for subsequent fragmentation and fractionation. The relative purity of the chloroplast suspension was determined by microscopical observation. Figures 2 and 3 are representative fields of the filtered homogenate and the WCI fraction, respectively.

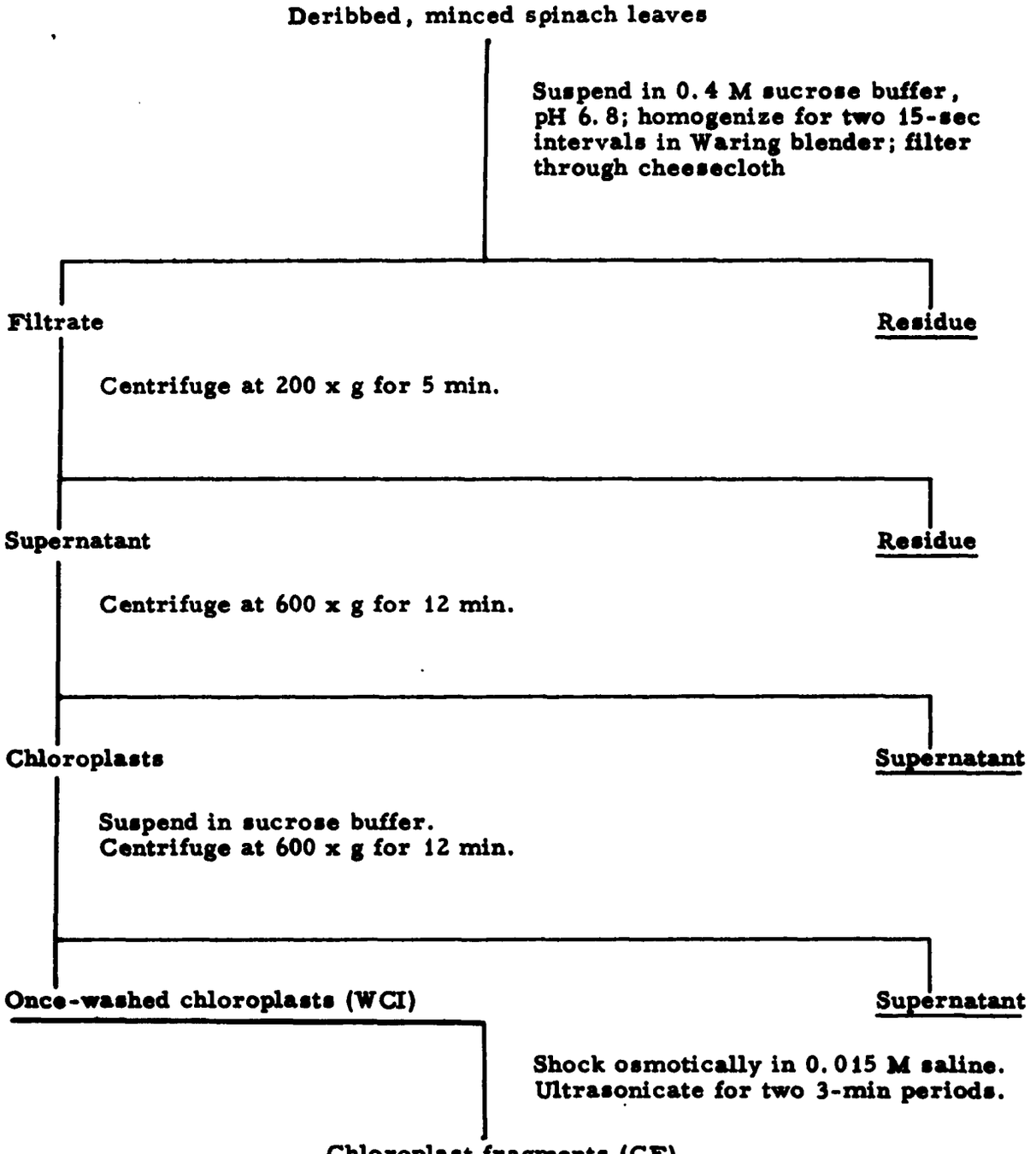
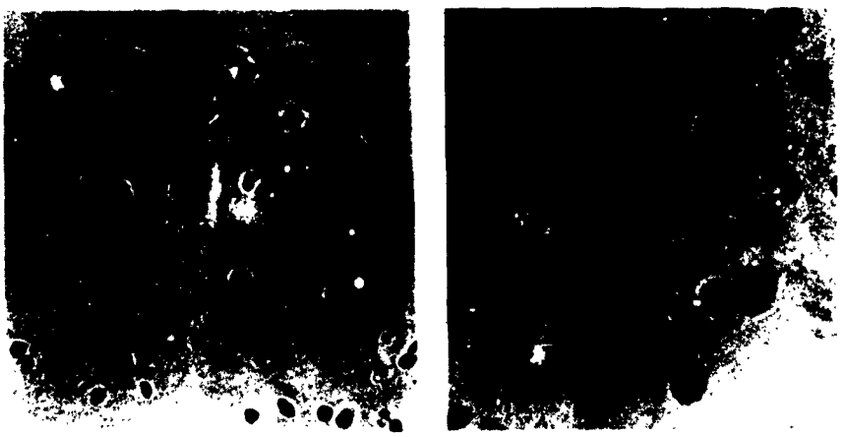


Figure 1

FLOW DIAGRAM FOR ISOLATING CHLOROPLASTS AND CHLOROPLAST FRAGMENTS

Figure 2

HOMOGENATE,
400 x



a - 400 x b - 1000 x

Figure 3

WASHED CHLOROPLASTS

WCI was resuspended in hypotonic saline (0.015 M sodium chloride) to a chlorophyll concentration of 0.08 to 0.2 mg/ml and subjected to ultrasonication in a Raytheon 9-kc 50-watt magnetostrictor. The fluid volume used in a single sonication ranged from 30 to 42 ml in various experiments. The vibration regimen found to be most suitable was two 3-min periods at the highest possible output with a 1- to 4-min rest period between treatments. The rest period was required to cool the suspension and maintain it between 8 and 12 °C.

Chloroplast fragments (CF) are designated by a subscript which indicates the treatment or the centrifugal force field under which they were sedimented. Thus, CF_{os} refers to the osmotically shocked, ultrasonicated fragments, and CF_{0-1} is the sediment obtained by centrifuging between 0 and 1×10^3 g. After ultrasonication, the fragment mixture (CF_{os}) was centrifuged at 1×10^3 g for 10 min to remove intact chloroplasts, large fragments, and agglomerates (CF_{0-1}). The supernatant was then differentially centrifuged, depending upon the experimental requirements, in the Spinco model L preparative ultracentrifuge under refrigeration. For forces up to 105×10^3 g, the number 30 rotor was used; for forces up to 145×10^3 g the number 40 rotor was used; and to reach forces up to 173×10^3 g the SW39L rotor was used.

III. ASSAY METHODS

A. Chlorophyll

Chlorophyll concentration was measured by the method of Arnon,[4] based on the specific absorption coefficients of chlorophylls *a* and *b* in 80% acetone as determined by MacKinney.[29]

B. Total Nitrogen

The procedure adopted for the measurement of nitrogen is a modification of the method of Willits, Coe, and Ogg.[43] From 2.0 to 8.0 ml of sample containing 2.0 mgms of protein is digested for 4 hr in micro-Kjeldahl flasks. In addition to the sample, each flask contains 1.30 gms of potassium sulfate, 0.04 gm of mercuric oxide, and 2.0 ml of 36 N sulfuric acid. The digested sample is dissolved in 8 to 10 ml of distilled water, and the digestion flask is attached directly to an all-glass micro-Kjeldahl distillation unit. Distillation is started after the addition of 8.5 ml of 50% sodium hydroxide containing 5% sodium thiosulfate. A quantity of 20 to 25 ml of ammonium hydroxide distillate is collected in 5.0 ml of 4% boric acid containing 4 drops of a mixed indicator (0.2% bromcresol green:0.2% methyl red, 5:1 by volume). Then 30 ml of distilled water is added to the distillate, which is titrated to a color end point with 0.01 N hydrochloric acid. The volume of hydrochloric acid used is related to nitrogen concentration.

C. Protein Nitrogen

Protein nitrogen is determined by mixing 4 vol. of sample with 1 vol. of 50% trichloracetic acid for 30 min at 5 °C. The mixture is centrifuged at 2000 rpm for 15 min in an International PR-2 centrifuge, and the supernatant, which contains less than 5% of the total nitrogen, is decanted. The residue is briefly washed with 12 ml of distilled water and centrifuged. The final residue is solubilized in sufficient 1 N potassium hydroxide to make the sample up to its original volume. The solution is stored at room temperature until it is assayed by the procedure for determining total nitrogen.

Chlorophyll-containing complexes can be recovered in the washed trichloracetic acid residue along with the protein constituents. About 6.23% nitrogen is present in chlorophyll, so that a calculation of the amount of protein nitrogen present requires subtraction of the chlorophyll nitrogen from the total nitrogen in the residue. Chlorophyll:protein nitrogen ratios were calculated in this manner.

D. Electron Transfer

Electron transfer was measured according to the procedure of Jagendorf and Krogmann.[23] Whole chloroplasts or chloroplast fragments which were resuspended in a given medium were assayed in their respective medium buffered at pH 6.8 with 0.05 M phosphate. Thus, chloroplast fragments which had been isolated in 0.015 N sodium chloride were assayed in buffered saline of the same concentration. Fractions for assay were added to the reaction mixture so that a total of 0.025 to 0.030 mgm of chlorophyll was present. The initial concentration of potassium ferricyanide, the oxidant, was 3×10^{-4} M. This level of oxidant allows the reaction to proceed so that the reaction rate is neither dependent on the oxidant concentration nor limited by the reduction step.[39] The resulting oxidant:chlorophyll ratio on a molar basis was thus about 30:1.

The entire procedure was carried out under subdued green light, except for a 1-min white-light illumination period at 800 ft-candles in a 15 °C water bath. Spectrophotometric readings were made at 510 mμ 5 min after o-phenanthroline was added to produce a color complex with free ferrous ions. Control reaction mixtures were not illuminated. All assays were run in duplicate. The standard curve used for calculation of micromoles of electrons transferred is based upon potassium ferrocyanide and ferrous sulfate standards as shown in Fig. 4.

E. Oxygen

Oxygen evolution was measured manometrically in a Warburg-type illuminated respirometer with single- or double-sidearm flasks. The reaction was run at 15 °C, 120 shakes per minute, and 800 ft-candles light intensity. Each flask contained a sample equivalent to at least 0.15 mg of chlorophyll. The oxidant, potassium ferricyanide, was added in excess so that the reaction would be independent of the nature and the concentration of oxidant.[39] The final concentration of oxidant was adjusted to 1.5 to 2.0×10^{-3} M giving a molar ratio of $\sim$ 30:1 oxidant to chlorophyll. The total reaction volume was made up to 3.0 ml with a buffer solution of 0.015 M sodium chloride and 0.05 M phosphate, pH 6.8. Both the center well and one sidearm contained 0.4 ml of Warburg No. 9 buffer mixture in order to maintain a constant partial pressure of carbon dioxide.

Prior to an experimental run, all the flasks were flushed for 15 min with oil-pumped nitrogen while shaking. After the gassing period, the stopcock and the sidearm were closed and the flasks were shaken for an additional 15 min before the oxidant was added from the sidearm. The flasks were then further equilibrated for 5 min, a zero reading was made, and the lights were turned on. Readings were taken every 3 min without cessation of shaking. The reaction was carried out for as long as 24 min, but after 15 to 18 min the rate of oxygen liberation generally decreased. Calculations for the rate of oxygen liberation were corrected for changes in the thermobarometer and the respective dark controls, and were based upon the rate of the reaction between the 6- and 18-min readings.

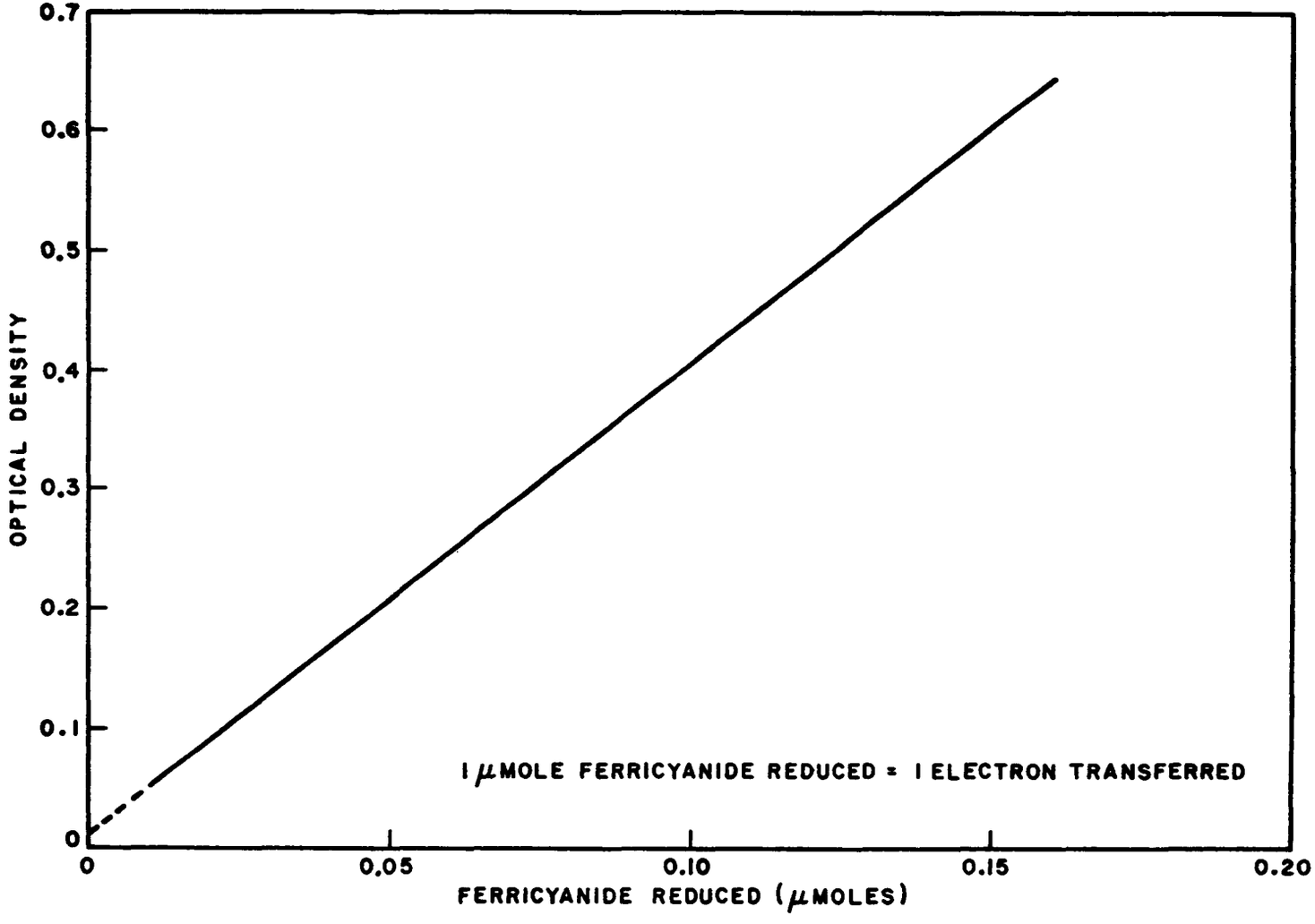


Figure 4

STANDARD CURVE FOR REDUCTION OF FERRICYANIDE

Theoretical oxygen yields were calculated from the stoichiometry of the electrochemical equation:[38]

$$4K_3Fe(CN)_6 + 4K^+ + 2H_2O \longrightarrow 4K_4Fe(CN)_6 + 4H^+ + O_2$$

Since 4 μmoles of electrons are transferred per μmole of oxygen and there are 22.4 μl of oxygen per μmole at standard temperature and pressure, theoretically 5.6 μl of oxygen should be liberated from each μmole of electrons transferred.

F. Plastoquinone

Methods for extraction of plastoquinone from chloroplast material have been based mainly on the work of Crane et al.[10-13] In preliminary experiments "wet" chloroplast fractions were extracted with isooctane. Crude extracts in a 1:1 ethanol-isooctane mixture were assayed directly by spectrophotometry, or were bulk-fractionated on Decalso, dried, dissolved in ethanol, and then assayed spectrophotometrically. The concentration of plastoquinone in each fraction was calculated from the equation:

$$\Delta E^{1\%}_{1\,cm,254} = \Delta E_{254} - (0.46 \times \Delta E_{275})$$

where

ΔE is the oxidized-reduced change of the spectrophotometrically measured extinction value at the wavelength indicated in the subscript;

$\Delta E^{1\%}_{1cm,254}$ is 198 for plastoquinone at its oxidized peak of 254 mμ;

0.46 is a constant to correct for the presence of coenzyme Q_{10}.

In other exploratory experiments, isooctane-extracted chloroplasts were lyophilized and assayed for photoactivity before and after the ethanol solubles in the extract were added to reconstitute the system.

In the light of recently reported information,[9,14,28] the extraction technique will necessarily be modified in future studies. Isooctane is no longer considered the solvent of choice, nor is extraction from completely dry material desirable; some water is required for maximum efficiency.

G. Cytochrome

Chloroplasts were extracted with 2% digitonin at room temperature for several hours according to the procedure of Gross and Wolken.[21] The digitonin-extracted pigment-protein complex was then fractionated with acetone and ammonium sulfate over a period of several days. The precipitate from the 0.45 to 0.90 saturated ammonium sulfate fractionation was dialyzed and concentrated by lyophilization. The concentrated material was taken up in dilute phosphate buffer, pH 7.0, and examined spectrophotometrically for the presence of cytochrome in the oxidized and reduced states.

IV. EXPERIMENTAL RESULTS AND DISCUSSION

A. Preliminary Studies

Pilot studies were conducted to ascertain the critical variables and the basic parameters which might affect the course of the investigation.

1. Leaf Storage

Washed, deribbed spinach leaves were stored either whole or chopped at -40 and +4 °C for 3-1/2 weeks. Periodically, aliquots of leaves were taken from each set of conditions, chloroplasts were isolated in 0.35 M saline,[5] and oxygen evolution and electron transfer activity were assayed.

Isolated chloroplasts from whole spinach leaves stored at 4 °C retained most of their original photoactivity for as long as 2 weeks. Decay of activity was hastened by mincing and by low temperatures. From these results, the storage methods described in Section II were determined.

2. Homogenization

Various methods have been reported for breaking leaf tissue in order to isolate chloroplasts. Grinding in cold sand with a mortar and pestle and homogenization in a Waring blender were both tried. The latter method was selected. The choice was based on microscopical observations of the chloroplast preparations (Fig. 2 and 3) and on the large quantity of tissue that could be handled by this procedure.

3. Fragmentation

The procedure for fragmentation of chloroplasts was designed to obtain sufficient quantities of material in the differentially centrifuged fractions for physiological and chemical measurements. Several techniques were tried. The efficiency of each method was determined by assaying for chlorophyll and photoactivity in each fraction.

No special effort was made to keep the suspensions anaerobic even though Thomas et al. reported activity loss in aerobically maintained chloroplast fragments.[41] There was no appreciable loss of activity in our experiments.

When chloroplasts were resuspended in a hypotonic solution to cause fragmentation by osmotic shock, 98% of the chlorophyll and activity was found in the fraction sedimenting at 1×10^3 g, indicating that little breakage had occurred. Various methods of mechanical rupture were then applied. The chloroplasts were ground with cold sand by using a mortar and pestle, homogenized in the Virtis "45" Omnimixer, or disintegrated in an ultrasonic vibrator. Ultrasonication for two 3-min intervals was found to be the most efficient means of fragmenting chloroplasts, and this procedure was routinely employed thereafter.

As shown in Table 1, the chlorophyll distribution obtained in this way was such that a sufficient amount for chlorophyll assay was present even in the extremely small particles sedimented at 173×10^3 g and physiological assays could be performed on the 145×10^3 g precipitate.

4. Media for Storage, Fragmentation, and Assay

Different investigators have employed a variety of isolation and fragmentation media for obtaining physiologically active chloroplastic material; the methods are listed in Table 2. The concern of this program with structure-function relationships of isolated fragments of chloroplasts necessitated an evaluation of the media and conditions.

To attain a desirable distribution pattern, ultrasonic fragmentation was found to be more efficient in a hypotonic solution (0.015 M saline) than in an isotonic solution (0.4 M sucrose buffer). In the hypotonic buffer chlorophyll was distributed more uniformly throughout the isolated fractions and electron transfer capacity was greater, as indicated in Tables 1 and 3. Isolation, fragmentation, and assay media were kept constant for each fraction. The one exception was for the intact chloroplasts, which were isolated and stored in isotonic solution and assayed in both isotonic and hypotonic solutions. The short exposure to osmotic shock in the hypotonic solution during the assay apparently had no effect on activity. For the other fractions, higher activity was elicited under hypotonic than under isotonic conditions.

5. Differential Centrifugation

After the chloroplasts were fragmented by ultrasonicating the osmotically shocked suspension, fractions were obtained by differential centrifugation in the Spinco model L preparative ultracentrifuge. Each sediment was suspended in 0.015 M saline to a measured chlorophyll content of about 0.3 mgm/ml for the assay of photoactivity by electron transfer and/or oxygen liberation. In some experiments aliquots were taken for protein determinations. A number of fractions from the same spinach batch were collected and assayed at the time of isolation of the fraction, after the last fraction was obtained, and again on the following day. In this way, any error introduced by a change in activity due to the time required to obtain all fractions was accounted for. Furthermore, the possibility of fractionating on one day and assaying the following day was also evaluated. The centrifugal data and the particle sizes in each fraction (estimated from the sedimentation data) are presented in Table 1. The chlorophyll distribution in relation to particle sizes in the centrifuged fractions is also shown.

Table 1

PARTICLE SIZE AND CHLOROPHYLL DISTRIBUTION IN DIFFERENTIALLY CENTRIFUGED FRACTIONS

Fraction	Maximum Centrifugal Force, g	Centrifugation Time at Speed, min	% of Total Chlorophyll	Estimated Particle Diameter, μ
CF_{0-1}	1×10^3	7-10	5.2	>3.0
CF_{1-10}	10×10^3	10	42.2	0.33-3.0
CF_{1-20}	20×10^3	10	55.8	0.23-3.0
CF_{1-50}	50×10^3	10	77.2	0.15-3.0
CF_{10-20}	20×10^3	10	13.7	0.23-0.33
CF_{20-50}	50×10^3	10	27.3	0.15-0.23
CF_{50-70}	70×10^3	30		0.10-0.15
CF_{50-105}	105×10^3	30	11.1	0.064-0.15
CF_{70-145}	145×10^3	30		0.047-0.10
$CF_{105-145}$	145×10^3	30	2.1	0.047-0.064
$CF_{145-173}$	173×10^3	30	1.5	0.032-0.047
$CF_{173,60}$	173×10^3	60	0.9	0.022-0.032
$CF_{173,120}$	173×10^3	120	0.4	0.012-0.022

Table 2

PROCEDURES FOR ISOLATING WHOLE CHLOROPLASTS AND FRAGMENTS

		Whole Chloroplasts		Chloroplast Fragments	
Reference	Assay	Isolating Medium	Osmotic Pressure, atm.	Isolating Medium	Osmotic Pressure, atm.
Spikes[37]	Hill reaction	0.50 M sucrose	11.5		
Spikes[38]	Hill reaction			0.50 M sucrose	11.5
Spikes[38]	Hill reaction			0.17 M sucrose[a]	2.8
Thomas[40]	Electron microscopy	0.15 M phosphate	6.7	0.30 M sucrose	6.9
Thomas[41]	Hill reaction			Water	1.0-1.5
Arnon[5]	Photosynthetic phosphorylation	0.35 M sodium chloride	14.7	Water	1.0-1.5
Jagendorf[22]	Photosynthetic phosphorylation	0.40 M sucrose	9.2	0.01 M potassium chloride buffer	1.0-1.5
San Pietro[34]	Photosynthetic phosphorylation			0.40 M sucrose	9.2

[a]For assay.

Table 3

PHOTOACTIVITY OF CHLOROPLASTS AND FRAGMENTS IN SOLUTIONS OF DIFFERENT TONICITY

Fraction	Activity, μmoles electrons per hr per mg chlorophyll		
	Isotonic		Hypotonic
	0.4 M Sucrose	0.35 M Sodium Chloride	0.015 M Sodium Chloride and 0.04 M Sucrose
Whole Chloroplasts	66.4	190.0	60.4
CF_s	113.2	98.0	110.0
CF_{1-20}	93.5	82.0	145.5
CF_{20-105}	46.6	2.2	130.7
$CF_{105-145}$			52.6

CF_s = chloroplast fragments, ultrasonicated.

6. Effect of the Supernatant

Since there is a time differential of several hours between the isolation and fragmentation of intact chloroplasts and the isolation of the last centrifuged fraction in any given experiment, it was necessary to determine the effect of exposure of particles to the supernatant suspension. The test was performed by resuspending an amount of the highly active fraction, CF_{20-50}, in the CF_{145} supernatant. The control was resuspended in dilute saline. After 20 to 24 hr of storage in the cold under these conditions, the Hill reaction was measured and found to be about 40% lower in the aliquot exposed to the supernatant. A similar experiment indicated that less inhibition occurred in the smaller particles of the $CF_{105-145}$ fraction.

B. Function and Particle Size

1. Rate of Photoactivity

The photosynthetic activity of each of the fractions was assayed by measuring electron transfer and oxygen evolution. Fractions isolated and resuspended in 0.015 M sodium chloride were tested 7 and 24 hr after isolation of intact chloroplasts. The pH of the suspensions was 6.2 to 6.4. Table 4 shows the correlation between oxygen evolution and electron transfer activity.

Table 4

COMPARISON OF ELECTRON TRANSFER AND OXYGEN EVOLUTION

Fraction	Electrons Transferred, μmoles per hr per mgm chlorophyll		Oxygen Evolved, μl per hr per mgm chlorophyll	
	7 Hr	24 Hr	7 Hr	24 Hr
CF_{os}	92	72	380	438
CF_{0-1}	86			
CF_{1-20}	105			
CF_{20-50}	138	157	522	563
CF_{50-70}		81		453
CF_{50-105}	74	60	410	
$CF_{105-145}$	75			
CF_{145spt}	28			

CF_{145spt} = supernatant from the $CF_{105-145}$ fraction.

The data demonstrate that the fractions remained stable for the test period. This is in direct contrast to the results of Thomas,[41] who reported decay of activity upon storage. Oxygen evolution parallels the pattern observed for electron transfer. The highest activity was found in the CF_{20-50} fraction. Lower activity is observed in all fractions isolated at centrifugal forces greater than 50 x 10^3 g. The establishment of these points after many tedious hours of experimentation has laid the groundwork for all subsequent studies and will serve as a baseline for studying the structural and the chemical changes responsible for the activity differential.

2. Effects of Chloride and Phosphate Anions

Jagendorf and Krogmann have shown that whole chloroplasts isolated in 0.4 to 0.5 M sucrose medium and diluted with 0.35 M sodium chloride at pH 6.3 have an electron transfer activity 5 to 10 times that for whole chloroplasts in sucrose alone.[25] The effect has not been clearly defined, but is thought to be related to permeability and an uncoupling of Hill activity from photophosphorylation.[24] Clendenning has studied activities of whole chloroplasts and "grana" preparations of various plants and has shown specific requirements for and specific effects of anions on photoactivity.[8] His studies also revealed a time-course effect. Gibbs and Calo demonstrated an optimal concentration range of phosphate for carbon dioxide fixation in whole chloroplasts; outside the optimum range phosphate was shown to have adverse effects and the measured reaction was minimized.[19]

As a result of these and other reported studies it became important to better define the ionic conditions for storing and assaying fragment preparations, to determine optimal conditions for each fraction, and thus to assure that activity changes were real. The effects of long- and short-term exposure and of changes in ionic conditions were examined at different sodium chloride levels by storing fragments in either 0.35 or 0.015 M sodium chloride prior to assay and then assaying in solutions of various salt levels. Table 5 demonstrates the experimental results.

Table 5

EFFECT OF SODIUM CHLORIDE ON PHOTOACTIVITY

Fraction	Sodium Chloride Concentration, M: Storage Solution	Sodium Chloride Concentration, M: Assay Solution	Electrons Transferred, μmoles per hr per mg chlorophyll: 3-4 Hr[a]	Electrons Transferred, μmoles per hr per mg chlorophyll: 20 Hr[a]
CF_{os}	0.015	0.015	70	68
		0.060	79	47
		0.35	94	70
CF_{1-50}	0.015	0.015	139	171
		0.060	111	131
		0.35	89	100
CF_{1-50}	0.35	0.015	206	58
		0.35	83	42
CF_{50-105}	0.015	0.015	60	97
		0.060	97	59
		0.35	135	113
CF_{50-105}	0.35	0.015	109	62
		0.35	70	22
$CF_{105-145}$	0.015	0.015	96	66
		0.060	103	54
		0.35	108	111
$CF_{105-145}$	0.35	0.015	84	42
		0.35	56	37

[a]Time of storage.

The rate of decay of particles stored in 0.35 M sodium chloride was, in general, greater and more variable than that of similar particles stored in the dilute saline solution. While there appeared to be some stimulation of the larger, more active particles (CF_{1-50}) stored in 0.35 M sodium chloride, this effect was not as pronounced in the smaller less active particles. The advantage of activation, however, is overshadowed by the disadvantage of the increased decay rate over a 20-hr period. Therefore, maintenance in the dilute salt solution is preferred.

High levels of sodium chloride in the assay solution appeared to enhance the activity of small particle fractions (CF_{50-105} and $CF_{105-145}$) and inhibit the activity of the large fragments (CF_{1-50}) when the storage solution was dilute saline but not when the storage solution was 0.35 M saline. This effect must be explored further.

To account for the fact that the conditions for optimal activity of small and large particles are different, a structural and/or chemical alteration is assumed. That a chemical difference exists is pointed out in Section IVC-1.

Table 6 shows the effect on broken chloroplast fractions of short exposure to various levels of phosphate in the assay solution. In both the highly active fraction CF_{1-50} and the less active fraction CF_{50-105} the pattern of effect was similar. At certain low levels of phosphate the ability to transfer electrons upon illumination decreased significantly. Above or below these levels, activity again increased. A clear explanation of this characteristic dip cannot be made. Jagendorf[24] and Good[20] have reported specific effects of cations and anions on potassium ferricyanide reduction in the Hill reaction. Such effects were found to stimulate or suppress the reaction in whole chloroplasts. Specific concentration ranges which resulted in decreased activity were described. On either side of these levels higher activity occurred. These peculiar responses to ionic changes appear to be reproducible under carefully controlled conditions. The effect is a fact; an interpretation of the effect is not possible at this time.

C. Chemistry and Function

1. Protein Determinations and Chlorophyll:Protein Ratios

The data in Table 7 indicate the averaged chlorophyll:protein nitrogen ratios obtained from a number of experiments. Total chlorophyll was measured, i.e., the sum of chlorophylls a and b. The ratio between the two chlorophylls in spinach chloroplasts is about 3:1. Figure 5 shows a typical absorption spectrum in 80% acetone of total extracted chloroplast pigments; the chlorophyll a and b absorption maxima are also indicated. Whether both these chlorophylls are functional in the photosynthetic mechanism is unknown. Consequently, the total chlorophyll concentration is assayed and used in calculations and in the construction of models.

Table 6

EFFECT OF PHOSPHATE ON PHOTOACTIVITY

Fraction	Phosphate Concentration, M	Electrons Transferred, μmoles per hr per mg chlorophyll
CF_{1-50}	Standard[a]	109
	0.1	116
	0.05	91
	0.01	44
	0.005	73
	0.001	73
CF_{50-105}	Standard[a]	75
	0.1	79
	0.05	70
	0.01	66
	0.005	44
	0.001	79

[a]The assay diluent: 0.05 M phosphate and 0.015 M sodium chloride.

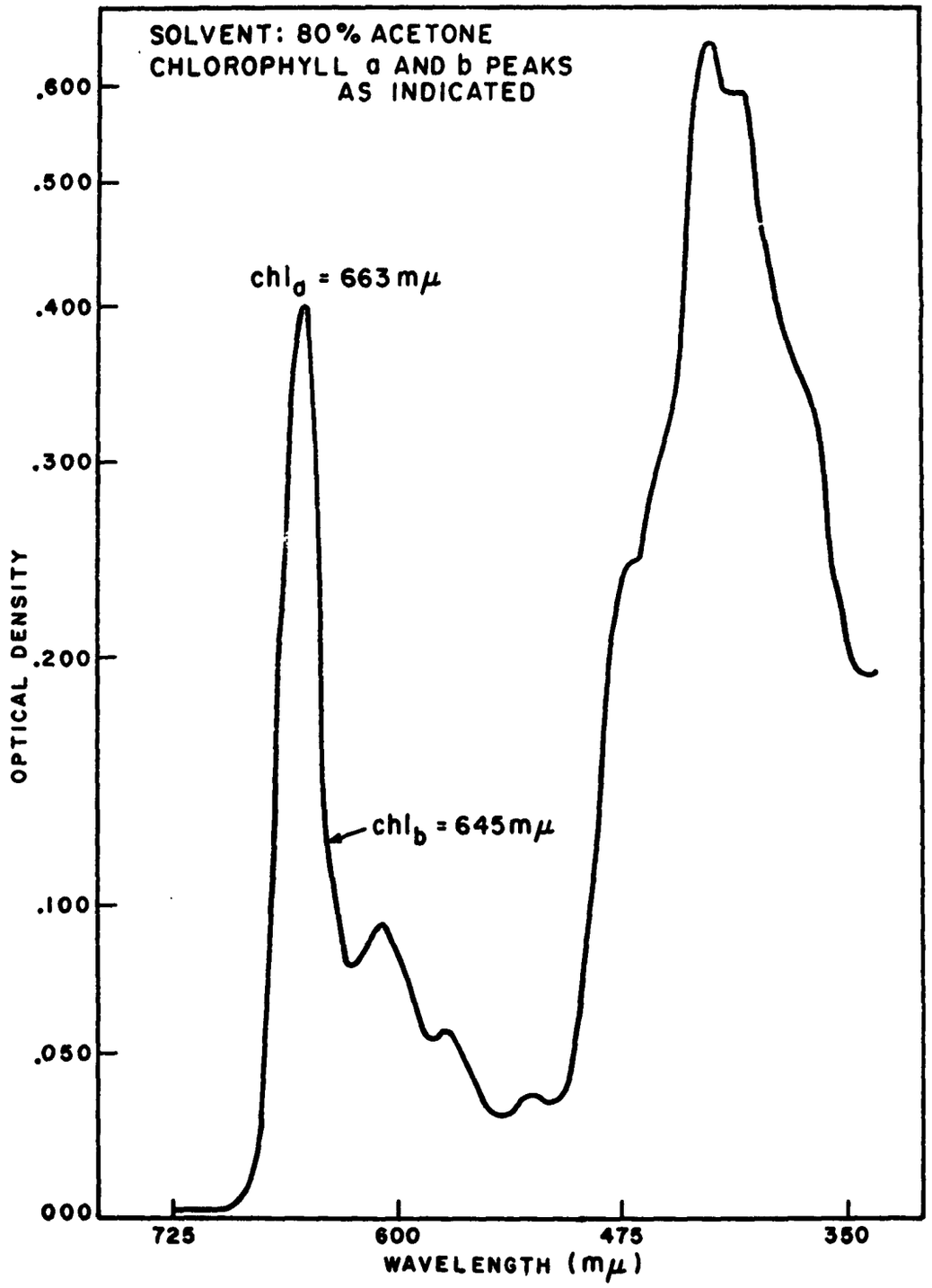


Figure 5

TYPICAL ABSORPTION SPECTRUM OF CHLOROPLAST PIGMENTS.

Table 7

CHLOROPHYLL:PROTEIN RATIOS

Fraction	Weight Ratio of Chlorophyll to Protein Nitrogen	Mass Ratio of Chlorophyll to Protein[a]	Protein in Molecular Weight Units per Chlorophyll Molecule[b]
Homogenate	0.21	0.0336	26,800
CF_{os}	0.76	0.121	7,450
CF_{0-1}	1.02	0.163	5,500
CF_{1-50}	1.39	0.223	4,040
CF_{1-20}	1.57	0.251	3,590
CF_{20-50}	1.27	0.201	4,480
CF_{50-70}	0.98	0.157	5,730
CF_{50-105}	0.78	0.125	7,200
CF_{70-145}	0.41	0.066	13,600
$CF_{105-145}$	0.38	0.061	14,750
CF_{145spt}	0.044	0.077	128,000

[a]Protein calculated by multiplying protein nitrogen by 6.25.

[b]Average molecular weight of chlorophyll taken as 900.

Measurement of the protein nitrogen content does not take into account the obvious possibility that onlycertain proteins in a preparation are actually associated with the functional chlorophyll molecules. In practice total nitrogen is measured and a correction is made for the nitrogen content of chlorophyll to obtain an estimate of protein nitrogen. Consequently, the results presented should be considered as approximations until sufficient information is obtained which might permit a more precise definition of the relationships between the chemistry, the activity, and the configuration of components in a basic photosynthetic unit.

The chlorophyll:protein ratios of isolated fractions of broken chloroplasts reveal an increase from the value in CF_{os} to CF_{1-20} and then a decrease (Table 7). The greatest ratio of chlorophyll:protein is not in the fraction of optimal activity. Possibly the specific chlorophyll:protein ratio in CF_{20-50} is responsible for the high activity of the fraction.

Depending upon the molecular weight assumed for the protein, the molar ratio of chlorophyll:protein in active chloroplasts is generally considered to be between 1:1 and 4:1. Compiled data of chlorophyll:protein mass ratios show values ranging from 0.053 to 0.33.[26,32] The calculated mass ratio for CF_{os}, which is equivalent to a whole chloroplast preparation, falls within this range; the ratio is 0.121 (Table 7).

Mass ratios are used to calculate the molecular weight of protein associated with one chlorophyll molecule. By assuming an average molecular weight for the typical protein, a hypothetical chlorophyll:protein macromolecular complex can be calculated. This technique was applied to the CF_{20-50} fraction by making the assumption that the average protein molecular weight is 10,000. From column 4 of Table 7 it can be seen that the molar ratio of chlorophyll:protein will thus be about 2. In other fractions the ratio will be either greater or less than this value. Relationships of this nature do not indicate the true weight of a protein molecule nor do they prove the existence of a basic photosynthetic unit. They are of value, however, in permitting a theoretical correlation of photoactivity with particle size and chlorophyll-protein organization.

2. Plastoquinone

Plastoquinone has been implicated in the electron transfer process in photosynthesis chiefly through Bishop's experimental work on chloroplasts.[7] When the quinone was extracted photoactivity was inhibited, and when the extracted material was reintroduced the system was reactivated. Whether the quinone is present and active in specific chloroplast fragments was not demonstrated. With this in mind, the following exploratory experiments were undertaken.

By following a modified technique for the "wet" extraction of plastoquinone, a chloroplast suspension in 0.25 M sucrose-phosphate buffer was shaken 1 to 2 hr with 4 volumes of isooctane. The suspension, centrifuged at 1×10^3 g, separated into three layers. The epiphase contained the yellow organic solvent, the aqueous hypophase contained green particles composed of intact and fragmented chloroplasts and aggregates, and the interface layer container particulate matter similar in appearance to the hypophase particles. These layers were separated and dried by lyophilization. The dried particulate phases were resuspended in the sucrose buffer and illuminated. Electron transfer activity was then measured. Similarly dried but unextracted chloroplasts served as the control.

The extracted particles exhibited about twice the activity of the controls, in contrast with Bishop's results. The addition of a measured amount of the dried epiphase extract in alcohol, calculated to replace the amount of material which had been extracted, did not influence activity. The total amount of plastoquinone which had been extracted was calculated (from the equation in Section IIIE) to be only 11% of the theoretical expectation. No clear-cut explanation for the apparent activation can be given at present.

The possibilities were considered that the isooctane extraction had been carried out for an insufficient period of time or that another solvent might be more effective. The extracted particles exhibiting increased activity were therefore pooled and again dried. One aliquot, serving as the control, was resuspended in the sucrose buffer. A second aliquot was reextracted with isooctane, separated, and again dried and resuspended in buffer. A third aliquot was extracted with petroleum ether and subsequently treated like the second aliquot. That the particles had lost activity due to the relatively rough treatment they had received was evident from their low specific activities. There was an indication, however, that reextraction with either of the organic solvents was inhibitory, although the low activities made the results somewhat questionable. These experiments must be repeated with a decreased time factor before the results can be interpreted.

3. Cytochromes

Several attempts were made to extract a c-type cytochrome from digitonin extracts of chloroplasts according to a method previously developed for Euglena.[21] The isolation of the c-type cytochrome, cytochrome f, from spinach chloroplasts has been reported.[15] The expectation that it might be found in a digitonin extract is based on the hypothesis that the cytochrome plays an integral role in photosynthetic oxidation-reductions which have been reported to occur in such extracts.[17,30]

In all cases spectral absorption scans gave negative results. The failure to identify the cytochrome by the procedure which was employed does not preclude its presence. The plant cytochrome f may be sufficiently chemically specific as to not be amenable to extraction by the method developed for Euglena.

Assays of oxygen liberation from digitonin extracts, aimed at verifying the photoactivity observed by others, have proved inconclusive in the few trial experiments attempted thus far.

D. Artificial Photosynthetic Systems

Whether there is a specific structural requirement for inducing the phenomenon of photosynthesis is debatable. In the natural system the lamellated organelle, the chloroplast, is the photoactive center. However, photoactivity has been shown to occur in structureless, uniform suspensions from digitonin-extracted chloroplasts.[17,30] Based on this work, studies were made of digitonin extracts of chloroplasts and of dried, layered chloroplast systems.

1. Digitonin Extracts

Digitonin is a non-ionic detergent which has been successfully used by others to extract a photoactive complex, chloroplastin, from chloroplasts. In our experiments isolated spinach chloroplasts were treated overnight at room temperature in the dark with 2% digitonin in 0.35 M sodium chloride. The extract was clarified by centrifugation at 20×10^3 g for 30 min in the cold, and aliquots containing 0.02 to 0.03 mgm of chlorophyll per ml were tested for electron transfer activity. The extract showed 1/100 the activity of the unextracted whole chloroplasts. The specific activity, though very low, was still measurable but of doubtful significance. Oxygen evolution was too low to be measurable by the standard manometric method.

These experiments were performed in the absence of added cofactors of photosynthesis, in the manner of all previous experiments performed under this program. The positive physiological activity noted by others was only in the presence of added cofactors. Additional studies will be necessary to ascertain the value and significance for photosynthetic studies of a nonstructured extract of chloroplasts.

2. Layered Chloroplasts

As a first approximation of a structured artificial photosynthetic system, the photoactivity of a layer of dried chloroplasts was tested. This seemed feasible, since French et al.[18] have demonstrated that chloroplasts maintain the ability to transfer electrons after being dried and Arnold and his associates[1-3] have shown that both dried chloroplasts and bacterial chromatophores are photoconductive.

In the experimental procedure, 1 ml of a chloroplast suspension with a predetermined concentration of 1 mgm of chlorophyll per ml was painted on the surface of a length of Visking dialysis tubing designed to accept a monolayer of chloroplasts when completely covered with the suspension. The films were air-dried and stored in a desiccator. The end of the painted dialysis sack was tied off and oxidant (potassium ferricyanide) in phosphate buffer was introduced into the tube. The open end was tied, and the sacks were shaken for 5 min in the dark or under illumination supplied by four 15-watt "cool white" fluorescent tubes. In addition to the dark control, one lacking oxidant and one not coated with chloroplasts were used. Chloroplasts were rinsed from the outside of the dialysis tubing after the illumination period but before the reaction solution was removed. This procedure effectively halted the reaction. The solution was then assayed according to the method of Jagendorf and Krogmann.[23]

In the controls without a chloroplast film or without oxidant present, no change in optical density at 510 mμ occurred, indicating that no electrons had been exchanged. The illuminated and dark experiments both displayed increased absorption, indicating that transfer of electrons across the semipermeable dialysis membrane had taken place. The apparent activity in the dark is assumed to have

resulted from an excessively high concentration of chlorophyll, which could have responded to the dim room light during the experimental procedure. The lack of a significant difference between the light and the dark samples may have been due to the total reduction of the added oxidant in both cases. Consequently, higher oxidant levels or lower chlorophyll concentrations seem necessary in future experiments.

V. THEORY AND CONCLUSIONS

The problem of duplicating a complex structure whose original construction was not observed and for which a blueprint is not available can be approached in two ways: (1) observe a similar structure or machine in the process of its manufacture, or (2) tear down the original piece by piece and painstakingly reconstruct it at each step of the procedure to assure that the original structural and functional integrity is regained. The latter approach, which is being employed in this study, places three requirements upon the investigator: (1) that he obtain all the available pertinent scientific facts, (2) that he consider the ideas of his contemporaries, and (3) that he use his imagination. These factors must be constantly reevaluated, placed in their proper perspective, and reorganized into a concept of the whole structure which can be eventually experimentally tested.

The classical report of Thomas et al. relating particle size to photoactivity in fragments of spinach chloroplasts indicates that particles about 75 A in diameter are the smallest particles expected to retain activity, while the "critical particle," almost twice as large, exhibits about 55% of the photoactivity of the control.[41] Whereas Thomas calculates that the critical particle could contain about 100 molecules of chlorophyll in a monolayer on its surface, we calculate that about 200 chlorophylls can be accommodated.

If the critical particle is considered to be a protein-chlorophyll macromolecular complex of the approximate size of Fraction I protein, a 200-A x 100-A oblate spheroid according to Park and Pon,[31] it should theoretically be coated with a chlorophyll-lipid monolayer over only one-half of its surface, as shown in Fig. 6. Since the surface area is twice that of Thomas' particle, 400 chlorophylls can be located on it, but only 200 on the upper half-surface. The volume of this particle, calculated from the formula for an oblate spheroid, would be 2×10^6 A^3, which is about twice the volume of Thomas' critical fragment. If the molecular weight of the protein portion of the pigment complex is assumed to be 10,000, each fully hydrated molecule would occupy a volume of 1.6×10^4 A^3. These calculations are based on a postulated composition of 80 amino acids, each 7 A to 8 A in height and 4 A to 5 A in diameter arranged as a folded accordion-chain or helix in an oblate spheroid shape of 40 A in diameter and 20 A in height. The oblate spheroid configuration of the macromolecule can then be ascribed to the shape of the component protein molecules and their intermolecular bonding.

The chlorophyll-lipid-protein complexes may involve different proteins, or some of the protein may not be complexed at all. Either supposition would explain the estimate that 200 to 400 chlorophylls are associated with a single cytochrome.[15,21] Since only one of the proteins in the macromolecule need be a cytochrome, applying the latter proposition it could be centrally located to act an an "energy trap" fed by the 200 surface chlorophylls.

For the sake of simplicity in visualizing the model, each protein member can be thought of as having the assumed average size, shape, and molecular weight. With room in the Fraction I protein macromolecule for a 30-A pigment-lipid surface layer, easily 75 but not more than 100 protein molecules would fit.

Thus the presumed chlorophyll:protein ratio of 2 derived from the mass ratio (Table 7) and the assumed molecular weight would be fulfilled.

By applying the same reasoning to the composition of Thomas' particle, about 50 proteins would fill its volume, thus also preserving the postulated ratio of 2. The weights of each of these macromolecules, calculated from the number of proteins and chlorophylls with some allowance for other components, would range from 0.5×10^6 to 1.25×10^6. These estimates are in close agreement with the report of Smith[36] for the weight of chlorophyll-holochrome, a particle extracted from chloroplasts, which is considered by many investigators to represent a basic photosynthetic unit.

Considering the data from this report in the design of the photosynthetic model, two points must be kept in mind: (1) the particles with optimal activity are estimated to be about 0.15 μ (1500 A) in diameter (Table 1); (2) the chlorophyll:protein molar ratio is approximately 2:1. On the assumption that this type of particle represents a small cylinder broken from a double lamellar disc, as if cut by a cork-boring tool, a cross section would reveal an upper and a lower chlorophyll-lipid surface. Each 30-A-thick layer is attached to the outer surface of a pair of overlapping Fraction I protein globules (Fig. 6). The top and bottom surface areas of the 1500-A-diameter cylinder, calculated from the formula for the area of a circle, are each about 1.6×10^6 A^2. The surface area which would be occupied by each Fraction I protein is about 4×10^4 A^2. Therefore about 40 of these protein units per surface, or a total of 80 units containing 1.6×10^4 chlorophyll molecules, would make up the 1500-A-diameter active particle. Tentatively this unit can be considered the optimal functional unit for Hill activity, composed of 80 basic structural units.

Similarly, 180 of the 130-A-diameter critical particles[41] could make up the optimal functional unit. Since each of these particles is layered with 100 chlorophylls, the 1500-A-diameter unit would have 1.8×10^4 chlorophyll molecules.

Either particle can fit the required conditions. However, the measured dimensions, shape, and weight of the oblate spheroid model[31] and the presumed ratio of chlorophyll to cytochrome which cannot be met by the 130-A-spherical particle point to the former as the model of choice at present. Until the intramolecular organization of this Fraction I protein is known, its acceptance as a basic unit remains conditional. Experimental evidence of the existence of such a structure and knowledge of its design are essential to solving the problem of building a photosynthetic system.

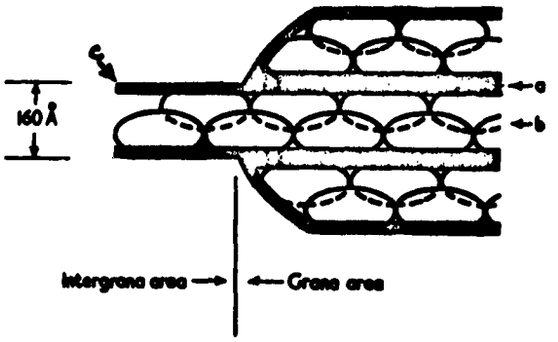


Figure 6

LAMELLAR MODEL OF SPINACH CHLOROPLAST
SHOWING OBLATE SPHEROID PROTEIN MACROMOLECULES
(After Park and Pon[31])

a - Pigment-lipid layer, 60 A thick
b - Protein layer, 100 A thick
c - Pigment-lipid layer, 30 A thick

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The structure, biochemistry, and photosynthetic function of the spinach chloroplast is being investigated to gain sufficient knowledge to permit a similar active system to be synthesized by man. Chloroplasts were isolated and fragmented. Relatively narrow-range size groupings of the fragments were obtained by differential centrifugation. These fractions were assayed physiologically and chemically. Maximal functional activity, in terms of electrons transferred or oxygen evolved under illumination, was found in particles sedimenting between 20×10^3 g and 50×10^3 g. Various anion concentrations were tested. An attempt to obtain photoactivity from a dried chloroplast layer--a first approximation of an artificial system--was only partially successful. The studies indicate that activity in the fractions varies, probably reflecting altered structural or chemical composition. Differences in protein nitrogen concentration and chlorophyll: protein ratios are evidence of chemical changes. Results are discussed in relation to proposed models for the fine structure of the chloroplast and the basic photosynthetic unit.

(over)

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1. Plants (Botony), photosynthesis, artificial
I. AFSC Project 6373, Task 637301
II. Contract AF 33(616)-7255
III. Armour Research Foundation, Chicago, Illinois
IV. Gross, J.A., Becker, M.J., Shefner, A.M.

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V. Secondary Rpt No. ARF 3174-17
VI. Aval fr OTS
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Aerospace Medical Division, 6570th Aerospace Medical Research Laboratories, Wright-Patterson Air Force Base, Ohio
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